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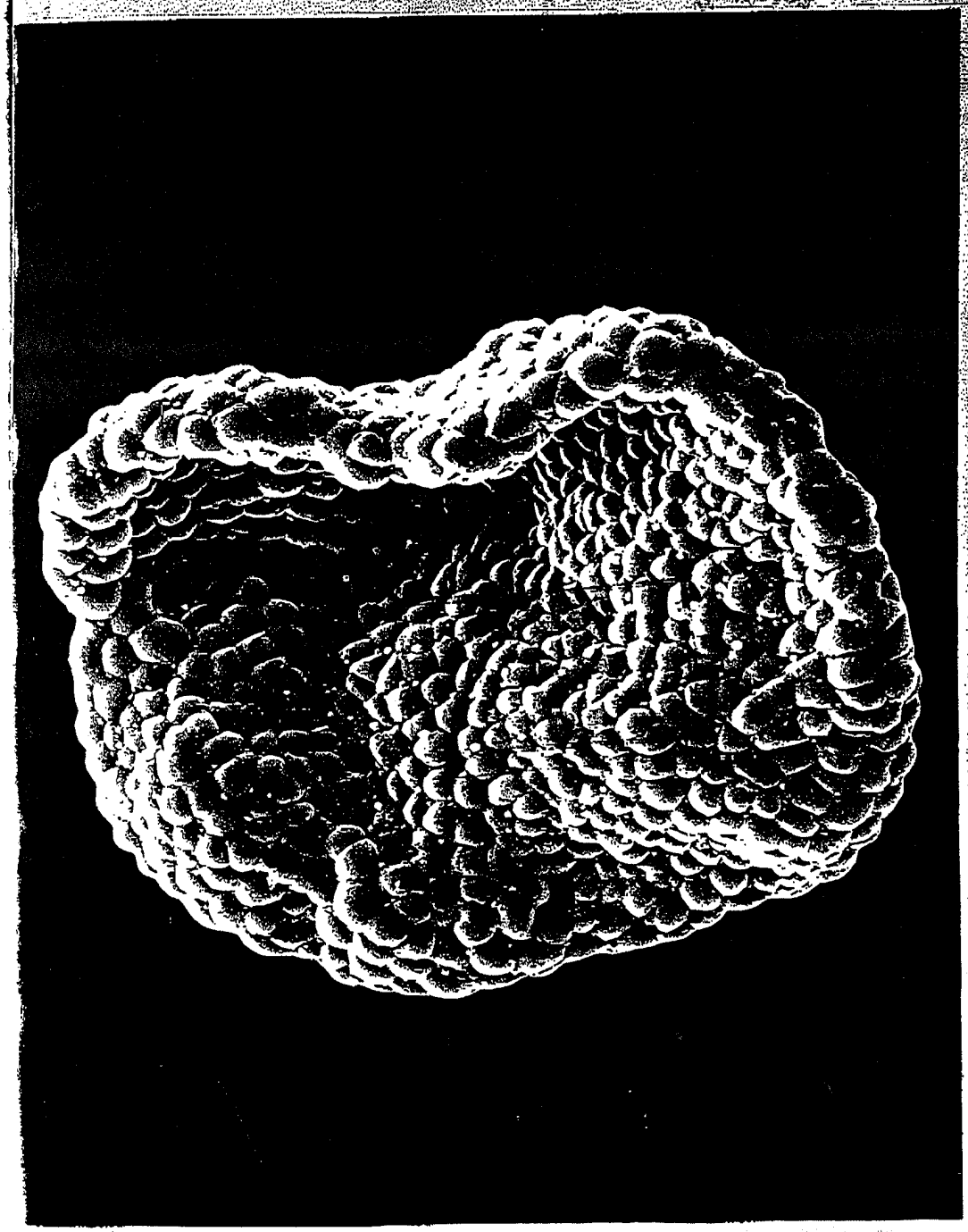

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Cover Photograph: Early embryonic stage of the coral, *Acropora millepora*. This planktonic stage, colloquially known as a prawn chip, floats passively, as cilia only appear later in embryonic development. See article by Miller and Ball, p. 291. Photo R. Heady & E. Ball.

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Notch signaling in the nervous system. Pieces still missing from the puzzle

Nicholas E. Baker

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Summary

Notch has been known for many years as a receptor for inhibitory signals that shapes the pattern of the nervous system during its development. Genes in the Notch pathway function to prevent neural determination so that only a subset of the available ectodermal cells become neural precursors. The localization of Notch signaling is crucial for determining where neural precursor cells arise on a cell-by-cell basis. The unresolved problem is that studies of the expression of Notch protein and its ligands are inconsistent with the pattern of neurogenesis. During neural cell fate specification, distributions of Notch protein and of its ligand Delta appear uniform. Under the reigning paradigm, such widespread expression should lead to N signal transduction in all cells and thereby prevent any neural specification. Yet, contrary to this expectation, neural elements still form, in characteristic patterns, hence, Notch signal transduction must have been inactive in the precursor cells. The mechanism preventing Notch signaling in certain cells must be posttranslational but it has not yet been identified. This review will outline the experimental evidence supporting this view of Notch signaling, and briefly evaluate some of the possible mechanisms that have been suggested. *BioEssays* 22:264–273, 2000. © 2000 John Wiley & Sons, Inc.

Introduction

In both vertebrates and invertebrates, neural precursor cells are specified from ectodermal tissues by virtue of bHLH regulatory genes. The proneural bHLH genes include three members of the *Drosophila* Achaete Scute gene Complex (*achaete*, *scute*, and *lethal of scute*), the unlinked *Drosophila* gene *atonal*, and their many homologs in other species.[1] Proneural gene expression defines domains where cells have the potential to differentiate as neural precursor cells. In normal development only some of the cells within these clusters progress to become neural precursor cells by expressing neural precursor genes (Fig. 1). Some neural precursor genes are also bHLH proteins such as *asense* from *Drosophila*, or neuroD from *Xenopus*.[1–3] Preventing most of the proneural cells from differentiating into neural cells is the role of receptor proteins of the Notch family. N signaling turns off proneural bHLH gene expression. Where this occurs, cells remain ectodermal and do not progress to neural specification. Neural patterning, therefore, depends on two opposing forces, the proneural bHLH genes driving neural determination, and Notch signaling opposing them in some cells, since neural precursor cells often arise intermingled with ectodermal cells, there must be a fine-scale pattern of N signaling on a cell-by-cell basis[4–6] (Fig. 1).

The N gene family encodes large transmembrane proteins with complex intracellular and extracellular domains. Their ligands are transmembrane proteins expressed on neighboring cells such as Delta. Recent studies suggest that Delta can also be cleaved to release its extracellular domain, which may potentially function in secreted form.[7,8] Many genes in the Notch pathway have been identified from genetic studies. Furthermore, similar pathways operate during insect and vertebrate neural development, as well as in the nematode *C. elegans*. Figure 2 summarizes what is known about the interaction of N with its ligands and how a signal transduction cascade is activated through proteolytic cleavage of the N protein.[9–11] Cleavage releases the N intracellular domain to enter the nucleus and act as a coactivator for the Su(H) protein, activating transcription of genes regulated by Su(H)-binding sites.[12] Chief amongst these targets are seven bHLH proteins encoded by the E(spl) gene complex.[13–16] In vertebrates, HES proteins are homologous to E(spl) bHLH proteins. E(spl) bHLH proteins and HES proteins are repressors that inhibit transcription of proneural bHLH genes.[17] They probably inhibit proneural gene function posttranslationally as well.[18,19] In addition, E(spl) gene transcripts may have the potential to form RNA:RNA duplexes with proneural gene transcripts, although the functional significance of this is not yet known.[20]

If the developmental pattern of neural precursor cells reflects a pattern of cells where N signal transduction remains inactive, how might this pattern arise? One possible mechanism is differential expression of Notch protein and its ligands (Fig. 3A). Cells lacking N protein could not inhibit their proneural genes, and cells lacking ligands could not activate N in their neighbors, so that an appropriate expression pattern of N and Dl would lead to an intermingled arrangement of cells where N signal transduction was active or inactive, hence to selection of particular proneural cells to

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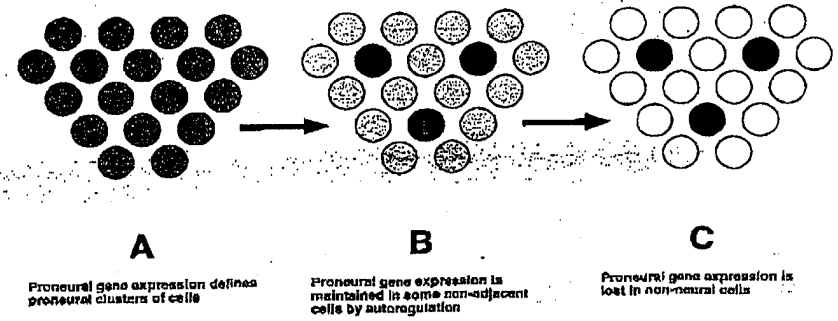

Figure 1. Three stages in the development of a proneural region. Shading indicates the level of proneural gene expression. A. Proneural regions of the ectoderm are defined by expression of proneural bHLH transcription factors. Proneural gene expression begins in all the cells in response to prepatterns. The prepatterns may have a distinct basis in each proneural region. B. Although proneural genes can be sufficient to define neural precursor cells, their expression is lost from many cells and maintained only in some. Maintenance usually depends on proneural gene autoregulation, and typically occurs in cells that are not adjacent to one another. C. Eventually the cells that maintain proneural gene expression are defined as neural precursor cells, whereas cells that lose proneural gene expression remain ectodermal. In neurogenic mutants such as *Notch* or *Delta*, all the proneural cells retain proneural gene expression and become neural precursor cells. Therefore neurogenic genes must be required to inhibit proneural gene expression in most of the cells. It has been inferred from the spacing of neural precursor cells that these are the source of signals that inhibit proneural gene expression in non-neural cells.

become neural precursor cells (Fig. 3A). Such regulated expression occurs in the *C. elegans* gonad.[21] Although not a neural tissue, cell fate specification in this two-cell equivalence group depends on a Notch homolog encoded by the *lin-12* gene, which is activated by the ligand encoded by *lag-2*. This system has served as a simple model for N function in neural tissues.[22] Indeed, some regions of the vertebrate nervous system may similarly utilize complementary expression of N and its ligands.[23] In this situation, N would be activated in proneural cells that express this receptor, whereas other cells would serve primarily as a source of the ligand. This could explain how certain cells undergo neural specification but not others.

One attractive feature of this model is its potential to explain the origin of intermingled patterns of neurogenesis. Since Lin-12-mediated signal transduction inhibits *lag-2* transcription and promotes *Lin-12* transcription, it is anticipated that neighboring cells will compete to express one gene or the other so that one becomes exclusively receptor-expressing and the other exclusively ligand-expressing.[21] Similar regulation of N and Dl transcription during neural specification might spontaneously lead to intermingled neural and non-neural cells.

Nevertheless, it has become increasingly difficult to consider the *C. elegans* gonad as a universal model for neural patterning, as study after study finds uniform N and Dl expression during neural specification in *Drosophila* tissues. The alternative is that in these tissues, N signaling must be prevented posttranslationally in cells where neural fate specification occurs. This essay reviews the evidence leading to this conclusion, and discusses some pathways that have emerged as candidates to regulate N signaling. How the putative post-translational regulation might be targeted to certain cells to produce intermingled patterns of neurogenesis will receive little attention in this review, although this question is clearly related to that of the molecular nature of the regulation itself.

Expression of Notch and Delta

Our starting point is that, in *Drosophila*, N and Dl appear to be evenly expressed in proneural regions (Fig. 4A,B), reflected in both RNA and protein distributions.[24] Although dynamic changes in single cells might be hard to observe, some of the studies reporting these results have quite carefully sought single cell resolution. Particularly well studied are the neuroblasts of the embryonic central nervous system,[25–27] the sensory organ precursor cells for the adult bristle organs,[28] and the R8 photoreceptor neurons of the developing retina.[29] The evidence shows that N and Dl proteins are actually widely and relatively uniformly expressed throughout proneural regions during *Drosophila* neurogenesis. For vertebrate neurogenesis, the picture awaits full description of the expression patterns of all the N and ligand proteins.

In addition to descriptive studies, other approaches have tested the importance of N and Dl expression levels experimentally. In one study of embryonic neurogenesis, Seugnet et al.[30] replaced either endogenous N expression or endogenous Dl expression with Gal4-dependent transgenes whose transcription was controlled by Gal4 expressed from other promoters. Neurogenesis proceeded relatively normally in such *Drosophila* embryos, suggesting that the precise pattern of N or Dl expression is not critical to the pattern of neural precursor cell specification.[30] Another study tested the role of differing N expression levels during neural determination in the *Drosophila* eye.[29] Genetic

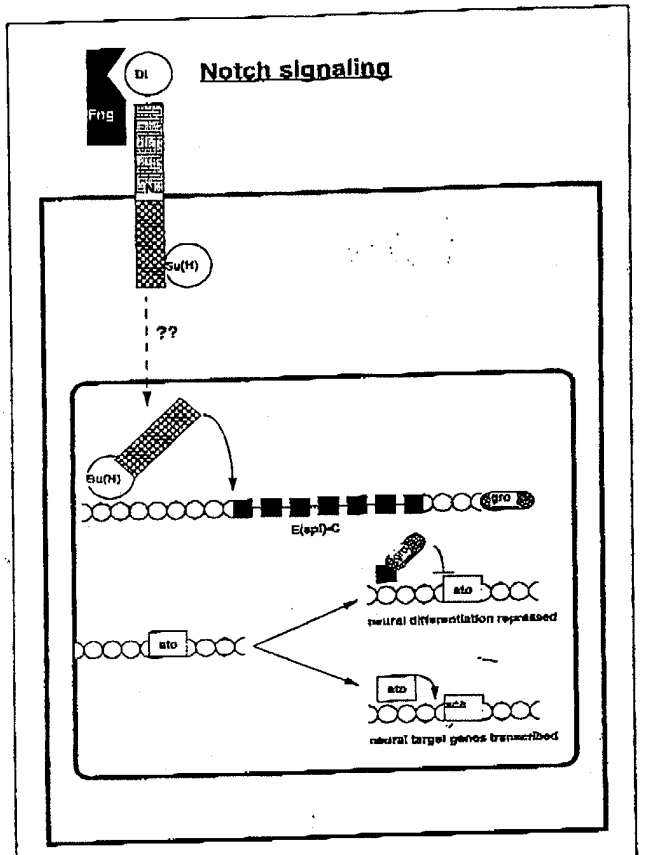

Figure 2. Notch signal transduction. The receptor protein N is activated by ligands such as Dl. At the wing margin, activation by Dl is promoted by the putative glycosyltransferase Fringe. Ligand binding leads to receptor proteolysis by an unknown mechanism that depends on presenilins.[11] The N intracellular domain enters the nucleus and complexes with the DNA-binding protein Su(H). This complex activates transcription of target genes including seven linked bHLH protein encoded by the E(spl) Gene Complex. E(spl) bHLH proteins are themselves DNA binding proteins that recruit the corepressor protein groucho to proneural gene promoters to silence them. Here the example shown is of *atonal*, the proneural gene for chrotonal organs and photoreceptor cells. Unless repressed by Notch signaling, atonal would proceed to activate target genes, one of which encodes the secreted protein scabrous, and to direct neural fate specification.

mosaics were studied in which adjacent cells differed either in the dosage for the endogenous N gene, or could be made to differ in N activity through the use of a temperature-sensitive allele. In the eye, neither different N gene doses nor different temperature-sensitivities of Notch protein function affected which cells were selected as the founder R8 photoreceptor cells. Again, this indicates that relative N levels on adjacent cells are not critical for deciding which cells take the R8 photoreceptor fate.[29] A third test involves ectopic ligand expression. One might predict that elevating ligand expression should activate N in additional cells and thereby reduce neural fate specification. This has not been observed. Neural precursor cells are correctly specified in the *Drosophila* adult peripheral nervous system even when they are adjacent to cells that overexpress ligands to high levels (our unpublished results). Taken together, all these experiments indicate that relative levels of N and Dl are not critical in deciding the spatial pattern of neural specification (Fig. 3A). Rather, they suggest that cells fated to become neural precursor cells maintain proneural gene expression because N signaling does not occur despite the presence of N on their surfaces, and of ligand on their neighbors' surfaces.

Even when uniformly expressed, N is differentially active

An alternative explanation is that another pathway is responsible for assigning neural cell fates, and that while N and Dl are required to inhibit proneural gene expression in non-neural cells, N signaling alone is insufficient to do this (Fig. 3B). In this situation, uniform Notch signaling might take place but would not be sufficient to prevent neural specification in certain cells. The evidence described below refutes this model and demonstrates that N signaling cannot be uniformly active in normal development.

Enough is now known about N signal transduction to permit experimental activation that is independent of ligand, and to assess the level of N signaling directly in vivo[9,10] (Fig. 2). Ligand binding and proteolytic cleavage release the N intracellular domain and expression of the intracellular domain is sufficient to produce ligand-independent signal transduction. Such artificial activation of N signaling has been found to prevent neural differentiation. This result strongly argues that neural cells would be inhibited by N signaling if they experienced it, and that in normal development N must not be activated in these cells.[13,31,32]

Other aspects of N signaling (Fig. 2) support the same conclusion. Major downstream targets of N signal transduction are seven bHLH proteins encoded by the E(spl) gene complex.[13–16] Since forced expression of E(spl) genes prevents neural precursor cell specification,[33,34] the implication is that activation of the N signal transduction pathway is sufficient to inhibit neural cell differentiation. Interestingly, particular E(spl) bHLH genes are expressed in different patterns, which suggests that N may not be the only signal activating transcription of these genes.[15]

If as these arguments suggest, N is not activated in certain neural precursor cells, then E(spl) genes should not be expressed in those cells either, and this seems to be the

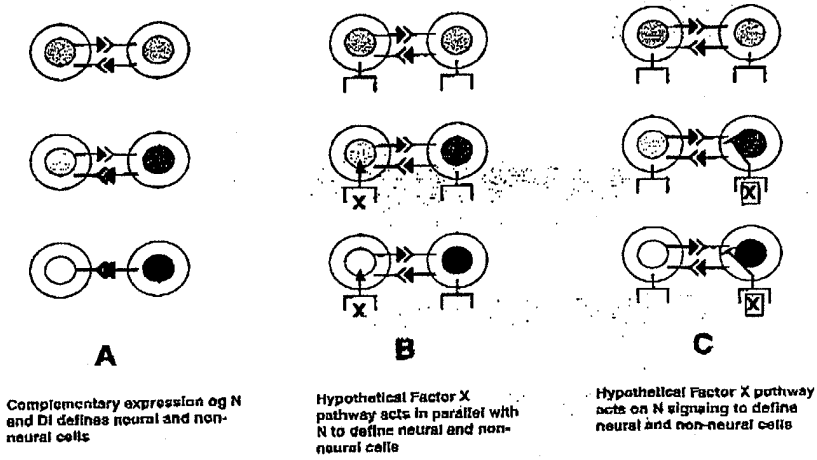

Figure 3. Three models for the specification of neural precursor cells. Shading indicates the level of proneural gene expression. **A.** The simplest model is based on studies of the *C. elegans* gonad. Two cells are initially equivalent and both express the receptor protein Notch and its ligand Delta. If levels of proneural protein fluctuate so as to increase in the cell on the right, this cell will start to express more Delta and less Notch. Such feedback amplifies the quantitative difference between the two cells until proneural gene expression is confined to the rightmost cell, which becomes the neural precursor. An attractive feature of this model is that the changes in Notch signaling and expression levels themselves select the neural cell. Evidence for this comes from proneural regions like the *Drosophila* thorax, where quantitative differences in *Notch* gene dose are indeed sufficient to determine the choice of neural cells.[5] Quantitative differences in *Notch* gene dose do not have this effect in all proneural regions.[29] **B.** In many proneural regions where lateral inhibition proceeds despite uniform distributions of N and Dl. N signaling might not be sufficient to inhibit proneural gene expression. A hypothetical signal X acts in combination with N to inhibit proneural gene expression and to inhibit neural cell fate. An alternative model (not shown) in which X acts in the neural cell to make them unresponsive to N signaling is formally equivalent. Both versions of this model are contradicted with several lines of evidence that suggest N signaling *is* sufficient to repress neural cell fate (see text). **C.** A third model is consistent with all the data known at present. A hypothetical signal X is shown acting on the future neural cell to block N signal transduction from occuring there. An alternative model (not shown) in which X instead acts on the non-neural cells to permit Dl to activate N signal transduction there is formally equivalent. This model does not automatically account for the spatial restriction of the signal X to particular cells. X may be controlled by N signaling in some way, to set up a competition analogous to that shown in Fig. 3A. Alternatively X may be localized by another pathway.

case (Fig. 4C,D). In normal development E(spl) expression occurs in proneural regions, but it is not detected in the neural precursor cells themselves.[14,32,35] Thus, all of the available data confirms the idea that it is the spatial pattern of N signaling (but not N RNA or protein levels) that determines neural determination. The pattern of neural precursor cell specification must be prefigured by an inverse pattern of N signaling because only in the absence of N signal transduction does specification occur. Some mechanism must exist that divides the population into cells where N either is or is not activated, despite apparently ubiquitous ligand (Fig. 3C), rather than overriding uniform N signaling (Fig. 3B).

Notch signaling activity can be regulated posttranslationally

Since N and Dl proteins are widely distributed, but N activation is restricted, it follows that there may be an additional condition required for N to be activated by Dl (Fig. 3C). The idea that Dl might not always be sufficient to activate N has a precedent from studies outside the nervous system, which address the role of N at the dorsoventral margin of the developing wing. Special marginal cells are defined by N signaling at the interface between dorsal and ventral cell populations.[36–40] N signaling is restricted to this interface even though N and its ligands are expressed more widely. N signaling is restricted in part through the action of the dorsally-expressed FRINGE (FNG) protein, which promotes activation of N by DL but antagonizes activation of N by SER.[41,42] This works to restrict activation of N to the boundary where dorsal (Serrate-expressing) and ventral (Dl-expressing) cells abut. Although no biochemical activity of FNG has yet been demonstrated, sequence features suggest it may encode a glycosyltransferase, one that presumably modifies N.[43]

Can Notch signaling also be restricted within proneural regions where N protein and its ligands are uniform? Analogous to the regulation by FNG which prevents SER from activating N in dorsal cells during *Drosophila* wing development, the activity of ligands, receptor, or both, must be regulated posttranslationally in the nervous system (Fig. 3C). The possibility that FNG itself is responsible has already been ruled out for neural tissues e.g., the retina.[44–46] Some other pathway perhaps acting analogously to FNG, or perhaps through a quite distinct mechanism, must be involved.

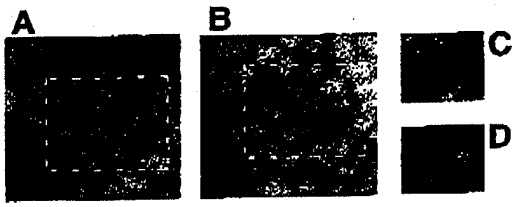

Figure 4. N and Dl expression in the retina. Panels A and B show the portions of the morphogenetic furrow region from the eye imaginal disc (anterior to the left). At this stage single R8 photoreceptor precursors have been selected by lateral inhibition. Yet levels of N protein **(A)** and Dl protein **(B)** on the apical cell surfaces are remarkably uniform. Dashed boxes approximately outline areas corresponding to proneural regions for each R8 cell. Panels **C** and **D** show confocal double images of the nuclear proteins atonal (green) and E(spl)mδ (red) two hours apart. The nuclei of about ten cells are visible in each panel, corresponding approximately to the dashed boxes in panels A and B. In D, E(spl)mδ (red) has replaced atonal (green) in all cells but one, indicating N signal transduction in all cells but the R8 precursor. Panels A, B and D correspond to the same stage. Data from Refs. 32, 39.

Different ways to initiate choice of neural cells?

Although the focus of this review is the mechanistic question of what prevents N activation in neural precursor cells, another question concerns how these cells are chosen. The mechanism that has been proposed for the *C. elegans* gonad involves complementary ligand and receptor expression through reciprocal effects of Lin-12 signaling on Lin-12 and Lag-2 transcription. This feedback loop leads to a competition to decide cell fate.[21] In neural tissues where N and Dl expression levels are uniform, and N and Dl function must be regulated posttranslationally, it is still possible for this to be downstream of levels of N signaling. Indeed, results of studies by Heitzler and Simpson[5] on the small innervated bristles (microchaeate) of the thorax show that initial levels of N gene dose affect choice of neural cells. Since levels of N and Dl proteins do not change,[28] initial levels of N signaling appear to affect the subsequent level of N or Dl protein function posttranslationally. By contrast, N gene dose has no affect on choice of R8 photoreceptor precursors in the retina. Other pathways might select specific neural precursor cells and impose this pattern on N signal transduction, e.g., through inhibition of N signaling in neural cells.[29] Regardless of variation in the impact of initial levels of N expression on choice of the neural cells between tissues, uniform N and Dl expression during specification indicates N or Dl protein activity as a target of the varied tissue specific patterning mechanisms.

Candidates for regulation of Notch signaling activity

How may the mechanism that restricts N signaling in the nervous system be recognized? Since there is no evidence for changes in levels of N RNA or protein during neural fate specification, it is reasonable to suggest that posttranslational mechanisms prevent certain cells from responding to N ligands. Mutants that block this protection would fail to specify neural precursor cells due to ubiquitous N activity. Because mutations in many genes might prevent neural fate specification for other reasons, such as mutations in proneural or neural precursor genes, an important additional criterion is that inappropriate activation of the mechanism should protect inappropriate cells, resulting in extra neural precursors. Finally, the proteins responsible for the pattern of lateral inhibition should be expressed or active in a pattern that predicts neural fate specification, since they in fact define this pattern. Although it is simpler to think in terms of a process that protects individual cells from N signal transduction, the reverse process, one that promotes N signal transduction in all the other proneural cells, would fit the data equally well.

Perhaps surprisingly, there is no shortage of candidates to define the pattern of lateral specification, provided by genes that fulfil one or more of the criteria of requirement, sufficiency, and pattern of expression or activity. The more difficult task is to decide which of these candidates actually define the pattern of neural specification during normal development.

The scabrous gene

One candidate, based on all three criteria, is the *scabrous* gene. *sca* encodes a secreted protein whose C-terminal 200 amino acids are related to the human blood clot protein fibrinogen.[47] However, no vertebrate protein homologous to SCA in its entirety has yet been reported. In *sca* null mutants, neural specification is perturbed so that the position of adult neural precursor cells becomes aberrant. This shows that the *sca* gene product is required to protect the appropriate cells from lateral inhibition. The *sca* expression pattern is exactly what might be expected for a gene that defines the pattern of Notch signalling. At first SCA is detected in all proneural cells, rapidly becoming restricted to the neural precursor cells. This mirrors the restriction of neural precursor fate and is consistent with SCA playing some role in this restriction.[48] When SCA is overexpressed or expressed ectopically, N signal transduction is suppressed.[49,50] Apparently, the extracellular SCA protein reduces the amount of N protein available for Dl or Ser. It has not been possible to detect direct binding of SCA and N proteins in tissue culture systems,[51] but there is now some evidence that the two proteins may interact under in vivo conditions (P. Powell and R. Cagan, personal communication).

There are problems, however, with the idea that *sca* alone is responsible for defining where inhibition will occur. One of the problems concerns the mutant phenotype. Many neural precursor cells still differentiate in *sca* null mutants, albeit in a chaotic pattern. This indicates that a mechanism for protecting neural precursor cells remains in *sca* null mutants, although its control may be awry, and suggests that *sca* might act indirectly to provide a proper environment or context for another mechanism to act, or is one of many genes that regulate N activity in neural cells. Another problem is that *sca* mutant phenotypes suggest that *sca* activates N signaling but ectopic expression inhibits it.[50] Full understanding of the role of *scabrous* in neural specification will require reconciliation of these facts.

Autonomous ligand action

A second candidate to inhibit N signaling is, paradoxically, the same Dl protein that activates N. Ectopic expression experiments show that in addition to activating N signaling in neighboring cells, ligands exert an autocrine inhibition on N signaling in the same cell. This phenomenon was first demonstrated outside the nervous system, during inductive signaling by N to define the dorso-ventral margin of the wing.[38,52] In this tissue autocrine inhibition of N signaling involving both Dl and Ser is important in normal development to restrict N signal transduction to cells at the interface between dorsal and ventral cells. At this boundary paracrine interactions occur; elsewhere autocrine interactions suppress N signal transduction.[53] This raises the possibility that whether N signal transduction occurs in any particular cell depends not on the presence or absence of ligands, which are widely distributed, but on a balance between paracrine activation of N signal transduction and autocrine inhibition of N signal transduction.

Although paracrine/autocrine balance seems an elegant mechanism for allowing cells that express the same molecules to be in different states, there are also several difficulties with such a model. First, it has not been possible to test definitively whether ligands are required to protect neural precursor cells from Notch signaling. This is because Dl is also required to activate N, hence without Dl, N is inactive and the possible mechanisms for preventing Notch signaling cannot be assessed. Second, as discussed above, N and Dl protein levels are remarkably uniform in most proneural regions, so some further explanation would be required to explain why autocrine or paracrine interaction predominates in distinct cells. If another pathway is required to achieve this, then perhaps that pathway is the actual determinant of neural patterning.

Receptor tyrosine kinase signaling

Another gene that can antagonize N signaling is the EGF receptor, a receptor tyrosine kinase. Like N, the *Drosophila* EGF receptor is involved in a large number of developmental decisions, as well as in growth control, and the pleiotropic effects of loss of EGFR function has made it difficult to dissect out all of its roles in particular processes.[54–56] Ectopic activation of EGF-R during bristle specification appears to antagonize lateral inhibition, resulting in extra-neural precursor cells (our unpublished results). Antagonism between EGFR and N pathways is well-known and may occur because of opposite effects of N and EGFR signaling on ETS-domain transcription factors. Nuclear ETS-domain proteins encoded by the *pointed* and *yan* genes act respectively as positive and negative effectors of RTK signaling in several *Drosophila* tissues. The same proteins play opposite roles in N signaling: Yan seems to be an effector of N signaling, which is antagonized by PNT-P2, a protein product of the *pointed* gene. Thus, EGF-R activates transcription by the PNT-P2 protein, while inhibiting the repressor action of YAN, which binds to similar DNA sequences. N signaling apparently involves the reverse.[57–59] Whether ETS-domain transcription factors are normally involved in lateral specification by Notch, however, is not yet known.

Two recent studies provide evidence that EGF-R function is normally required for one example of neural specification, that of the R8 photoreceptor cells in the developing eye.[60,61] Surprisingly, both studies indicate a requirement for EGF-R to *promote* inhibition, not to antagonize it. Equally surprisingly, two distinct mechanisms may be involved. It should be noted that there are also other interpretations of the role of the EGF-R during eye patterning,[62] full discussion of which lies outside the scope of this review.

One study used a specific mutant allele of the EGF-R which appears to arrest EGF-R activity at a level lower than that which normally occurs during eye development but it is not a null allele. This mutation prevents R8 specification by activating N signaling and E(spl) expression in all cells, including those which should normally become R8 precursors, hence preventing their differentiation. This suggests that low levels of EGF-R activity promote N signaling, a conclusion supported by analysis of EGF-R null mutant cells. The latter show prolonged proneural gene expression and a small increase in the number of R8 precursor cells specified.[61]

The other study also reported increased numbers of R8 cells in EGF-R null mutant clones, and took the analysis further to identify a target gene whose expression is EGF-R dependent.[60] This target is the homeobox gene *rough*. Null mutations in *rough* show similar effects on R8 precursor specification as EGF-R null mutant cells,[35] hence the ROUGH protein is required to restrict neural precursor selection, and is highly likely to promote N signal transduction in some cells.

It is intriguing that these two studies identified *E(spl)* (via *N*) and *rough* as two distinct targets of EGF-R signaling. In

normal eye development *E(spl)* and *rough* expression correspond very closely, suggesting some common regulatory control.[35] In addition, the pattern of cells where EGF-R is active (measured by antibodies against phosphorylated MAPKinase) is very similar to the pattern of cells where N is active (measured by antibodies against E(spl) proteins.[61–64] *Rough*, however, is required for inhibition of only a few cells, whereas E(spl) is required to prevent a ten-fold increase in R8 cell specification.[34] It will be interesting to determine how expression of E(spl) genes integrates other factors in addition to N signaling, such as EGFR signaling and homeobox genes. Distinct expression patterns of the E(spl) bHLH genes further suggest that factors besides N must be important. In addition, ROUGH protein might inhibit proneural gene expression independently of E(spl), by some unknown mechanism.

A different link between receptor tyrosine kinases and neural precursor selection has been suggested for vertebrate cells. The PC12 cell line undergoes neural differentiation in response to NGF. NGF interacts with tyrosine kinase receptors to activate Protein kinase C, which results in phosphorylation and inactivation of endogenous HES-1 protein. In the absence of NGF, HES-1 normally prevents spontaneous neural differentiation by PC12 cells.[65] Protein kinase C can be activated by other receptor tyrosine kinases and in principle this provides a mechanism by which receptor tyrosine kinase signaling in neural territories might block function of HES-1-like proteins, rendering N signaling ineffective and permitting neural fate specification in particular cells. Posttranslational regulation of E(spl) proteins in *Drosophila*, however, has yet to be reported.

Proneural cofactors

The idea of cofactors that might provide spatial specificity is not restricted to E(spl) and ROUGH. There is also evidence for cofactors in proneural gene autoregulation. Proneural gene expression begins in response to diverse signals, probably distinct for each region of the nervous system, collectively called "prepatterns".[66–68] Prepatterns are usually transient, and replaced in each part of the nervous system by autoregulatory proneural gene expression.[69,70] N mainly acts by blocking proneural gene autoregulation, and has less effect on prepatterns.[32,71,72] Recent studies suggest that proneural gene autoregulation may not simply reflect activation of proneural gene promoters by proneural protein binding, but must depend on other proteins as well.

One study identified an autoregulatory enhancer element for the proneural gene *scute*.[71] The element not only contains potential SC protein binding sites, but also another conserved sequence predicted to bind an unidentified NF-κB class protein. If spatially restricted, such a cofactor might give particular cells advantages in withstanding N signaling. Study of a second proneural gene, *atonal*, leads to a similar conclusion.[72] The *ato* gene has two separate autoregulatory enhancer elements, each sufficient for autoregulation in distinct subsets of the tissues where *ato* functions. Since neither enhancer works in all tissues expressing *ato*, ATO protein alone cannot be sufficient to activate in any tissue; at least two distinct essential cofactors must exist, one for each enhancer.

At present, none of these putative autoregulatory cofactors has been identified. In the case of certain embryonic neuroblasts, a homeodomain protein encoded by the *vnd* gene appears to promote proneural bHLH gene expression in future neural precursor cells. This occurs independently of proneural autoregulation, however, because a *vnd* promoter element is active in certain neural precursor cells even in embryos deleted for the AS-C.[73] Another protein that is known to promote neural cell specification is the product of the *Bearded* gene.[74] *Brd* encodes a novel protein and it is not known whether it acts as a transcription factor, or whether it promotes neural cell specification through proneural genes or independently of them. Null alleles of *Brd* have no phenotype and the gene may be redundant, possibly with the E(spl) genes m4 and mα, non-bHLH genes with which *Brd* shares sequence similarity.[75] The expression pattern of these genes is not known with sufficient precision, however, to say whether they might be responsible for defining the cells that will escape inhibition, and if so what signals would be responsible for establishing this pattern.

Multiple forms of Notch and Delta

There is increasing evidence that several forms of N and Dl might exist in vivo. In the case of N, proteolysis of the extracellular domain occurs in the trans-Golgi during synthesis, so that the mature protein contains the two associated proteolytic fragments.[76] This cleavage event may be mediated by a furin-type convertase.[77] Regulation of this cleavage process could, in turn, regulate N activity in neural cells, although there is no experimental evidence for this at present. There is, however, some recent evidence that other different proteolytic forms of N might be present in vivo and that these might differ functionally from one another.[78] Proteolysis of Dl has also been reported, resulting in a secreted form of the Dl extracellular domain as well as the familiar transmembrane-tethered protein.[7] The metalloprotease encoded by the *kuzbanian* gene is required but the functional consequences of Dl cleavage are not yet fully known.[8] Clearly, such modifications might lead to forms of N or Dl that prevent signaling in particular cells. For example, it has been suggested that dimerization is important for signaling by N proteins.[79] If this is correct, then modifications that prevent dimer formation could render particular cells unable to respond to ligands.

Intracellular components of the Notch pathway

A number of genes have been described whose products bind the intracellular domain of N, and whose mutations or misexpression alter the effectiveness of N signal transduction. These include *dishevelled*, *deltex*, and *Notchless* from *Drosophila*, and *emb-5* from *C. elegans*, and *bcl3* from human cells.[80–84] In addition, the Hairless protein binds to Su(H) and inhibits DNA binding.[85,86] Any one of these proteins might be important in the regulation of N activity. The critical question is whether the activities of any of these proteins is regulated so as to result in different levels of N signal transduction in different cells. The alternative is that many of these proteins only define a uniform cellular context in which N acts in all cells, without contributing to differences between them.

The wingless gene

Another secreted protein whose genetic interactions with N merit attention is the one encoded by *wingless*.[87,88] In some tissues, activation of WG signaling by overexpression of the intracellular protein dishevelled can promote neurogenesis, apparently by inhibiting N signaling.[82] It is not known whether WG signaling does this during neurogenesis in wild-type, but this remains an interesting possibility. Certain sensory bristles are known to require WG, but at an earlier stage, prior to N function.[89,90]

Conclusions

In this article, I have reviewed experimental evidence that activity of the N signaling pathway is posttranslationally regulated during neurogenesis in *Drosophila* and that, whatever the mechanism is, its spatial regulation must be a major factor in determining the pattern of neurogenesis. Posttranslational regulation of N signaling by the putative glycosyltransferase FNG proteins is already known from studies of other tissues, although not from the *Drosophila* nervous system. As components of the N signaling pathway are conserved in other organisms, it seems possible that the hypothesized posttranslational regulation of N signaling might also be conserved.

Interestingly, N would not be the first signaling pathway in *Drosophila* to be controlled by other components. Cell fate specification by the EGF receptor often depends on the TGF-α-like ligand Spitz, and since *spitz* and *EGFR* genes are widely expressed, patterning is achieved by restricted expression of proteins such as rhomboid, which are somehow required for ligand processing.[91]

Whereas genetic studies readily identified *rhomboid* as a gene required for spitz function and so led to identification of its role as a specificity factor in EGFR signaling, genetic studies have not yet clearly pointed to the mechanism by which neural precursor cells avoid N signaling in response to the Dl protein to which they seem to be exposed. Instead, a number of genes have been found that possess some properties consistent with a role in this process, and it is presently unclear which if any of them are actually responsible. Possibly some previously unstudied gene, whose neurogenic mutant phenotype has been obscured by a maternal effect, is responsible. It also seems possible that several pathways collectively contribute to the selection of neural precursor cells, so that the sum of modest differences in proneural protein levels, functional consequences of the stiochiometric ratios between N and D1 in proneural cells, and of the activity of other pathways such as receptor tyrosine kinases and SCA together lead to a situation in which N signal transduction remains insignificant in particular cells within proneural clusters. It remains to be seen whether these pathways collectively control the pattern of Notch signaling, or are less significant than novel mechanisms yet to be discovered. Until these questions are resolved, geneticists remain unable to say what causes certain proneural cells to undergo neural specification and others to be inhibited.

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Note added in proof

Several recent papers have further described processes that promote or counteract Notch signaling.

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